

CROSSING EFFECTS ON SEED VIABILITY AND
EXPERIMENTAL GERMINATION OF THE FEDERAL
THREATENED *PLATANThERA LEUCOPHAEA*
(ORCHIDACEAE)

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ABSTRACT. We conducted experimental pollination and seed germination trials with *Platanthera leucophaea*, a threatened north temperate grassland orchid species for which propagation and restoration are important conservation needs. Our objectives were to determine how the species' breeding system and modes of pollination interact to affect production of viable seed and seed germination, and how seed germination responds to stratification and to inoculation by mycorrhizal fungi. Outcrossing by hand pollination produced a higher percentage of viable seed than did natural pollination, as did outcrossing between populations in comparison to outcrossing or selfing within populations, indicating a facultative outcrossing breeding system. Outcrossing also enhanced percent germination, which was positively correlated with the percentage, but not number, of viable seeds. A 16 wk. stratification period resulted in significantly higher percent germination than 8 wk. stratification or no stratification. Germination was further enhanced by inoculation with a mycorrhizal fungus (*Ceratohiza* sp.) isolated from *P. leucophaea*. These results indicate that the breeding system of *P. leucophaea* allows for greater numbers of viable seeds with greater germination rates when plants are outcrossed. Thus, hand pollination and outcrossing can enhance propagation and restoration efforts, especially when coupled with scarification and stratification treatments that maximize germination rates.

Key Words: Orchidaceae, *Platanthera*, seed germination, breeding system, pollination, conservation

Gaining a better understanding of the propagation and restoration requirements of terrestrial orchids has become crucial to their conservation (Johansen and Rasmussen 1992; Zettler 1996a), and is an important conservation objective for the Federal Threatened eastern prairie fringed orchid *Platanthera leucophaea* (Nutt.) Lindl. (Bowles and Bell 1999; U.S. Fish and Wildlife Service 1999). This orchid occurs in tallgrass prairie remnants

and wetlands in eastern Iowa, southern Wisconsin, and northern Illinois and Indiana, and in shoreline prairies, sedge meadows, bogs, and fens eastward to Maine (Bowles 1983; Sheviak 1974; Sheviak and Bowles 1986). It has declined by more than 70% from original county records due to habitat conversion to agriculture. Remaining populations are often small and continue to be threatened by succession to woody vegetation, competition from exotic species, illicit collecting, and drainage (Bowles 1983; U.S. Fish and Wildlife Service 1999). In this study, we report on effects of experimental pollination on its seed production, and effects of seed treatment and fungal inoculation on seed germination.

Showy *Platanthera* are thought to have facultative outcrossing breeding systems (Gregg 1990), a well known strategy for avoidance of deleterious effects of inbreeding (e.g., Willson 1983). *Platanthera leucophaea* has the largest flowers and nectar spurs of eastern North American *Platanthera*, and its large floral display and lack of vegetative reproduction indicate a strong investment toward pollination and seed production (Bowles 1983, 1985; Sheviak and Bowles 1986). Pollination is by hawkmoths. The orchid pollinarium, which comprises a pollinium (pollen mass), caudicle, and viscidium, adheres to a hawkmoth's proboscis by the viscidium. Caudicle movement (taxis) positions the pollinium for contact with the stigmatic surface after about 40 seconds, thereby promoting outcrossing (Bowles 1985). Pollen grains are then removed from the pollinium as they adhere to the plant's stigma. Selfing or geitonogamy may occur when moths revisit flowers or inflorescences, especially in small orchid populations, and could potentially influence production of viable seeds if this species is affected by inbreeding depression. As in *P. praeclara* Sheviak & M. L. Bowles (Sieg and King 1995), most plants flower once and the median number of flowering seasons is less than three. Seedling establishment is therefore an important life history stage for this species, and pollination and seed production are critical factors in population viability (Bowles and Bell 1999).

Terrestrial orchids are difficult to propagate due to physiological seed dormancy and the need for mycorrhizal fungi for successful seed germination and seedling development (Rasmussen 1995; Stoutamire 1974; Zettler 1996b; Zettler and McInnis 1992). Experimental propagation with mycorrhizal fungi has been re-

ported for a few species of the widespread north temperate orchid genus *Platanthera*, including *P. integrilabia* (Correll) Luer (Zettler and McInnis 1992), *P. clavellata* (Michx.) Luer (Zettler and Hofer 1998), and *P. leucophaea* (Zettler et al. 2001). Seed germination was highly variable among seed sources in these studies, and was facilitated by, but not dependent upon, the presence of mycorrhizal fungi. Such variation may be influenced by many factors, including sensitivity to inbreeding and levels of genetic diversity within populations of different sizes (e.g., Fenster and Dudash 1994; Weller 1994), and differing germination requirements or different experimental methods used by researchers (Rasmussen 1995; Zettler 1996b).

Seed germination and mycorrhizal fungi of *Platanthera leucophaea* were first investigated by Curtis (1939), who isolated species of the soil fungus *Rhizoctonia* (now *Ceratorhiza*; Andersen and Rasmussen 1996) from *P. leucophaea* roots in different habitats. Curtis was unable to germinate seeds inoculated with these fungi, perhaps due to failure to properly scarify or stratify seeds. Stoutamire (1996) increased asymbiotic seed germination rates by increasing scarification time in diluted NaOCl, and recommended two or more months of cold stratification. Stoutamire (1996) also germinated *P. leucophaea* seeds in 35 m μ Nitex bolt cloth (following Rasmussen and Whigham 1993) buried in prairie sod that contained soil fungi, but neither mycorrhizae nor further seedling development occurred. Zettler et al. (2001) achieved mycotrophic germination of *P. leucophaea* seeds using a *Ceratorhiza* species isolated from roots of this orchid, with development of leaf-bearing seedlings occurring after a second cold treatment.

More information is needed about optimum pollination and germination requirements of *Platanthera leucophaea*, factors that will lead to a better understanding of its reproductive biology, population demographic processes, and restoration requirements. Our study had two related objectives. First, we assessed how different modes of pollination (i.e., natural versus hand pollination, selfing, outcrossing within, and outcrossing between populations) affect the percentage of viable seeds and the germinability of seeds. Because of the breeding system of this species, we expected that outcrossing would enhance seed viability by reducing inbreeding depression. Second, using scarified seed, we tested how stratification periods and symbiotic versus asymbiotic con-

Table 1. Seed sources, sample sizes, and seed collection dates for experimental crossing and germination of *Platanthera leucophaea*; n = number of plants sampled for seed.

Site	Location	Sampling Date and Sample Size by Experiment	
		Germination Experiment	Crossing Experiment
Abbott Park	Lake Co., Ill.	Aug 1996 (n = 3)	Sep 1997 (n = 5)
			Sep 1998 (n = 1)
Wadsworth Prairie	Lake Co., Ill.	Aug 1995 (n = 111)	Sep 1997 (n = 3)
		Sep 1996 (n = 30)	Sep 1998 (n = 18)
Lyons Woods	Lake Co., Ill.	Sep 1996 (n = 82)	
Pickerel Creek	Sandusky Co., Ohio	Oct 1995 (n > 10)	

ditions affected germination. We expected that longer stratification and symbiotic conditions would enhance germination.

MATERIALS AND METHODS

Seed and fungus sources. *Platanthera leucophaea* seeds were obtained from one site in Ohio and three sites in Illinois (Table 1). The Pickerel Creek, Ohio, site contains one of the largest known *P. leucophaea* populations, where plants occupy successional wetland habitat of the Lake Erie lake plain (U.S. Fish and Wildlife Service 1999). The Illinois sites are in Lake County, in the Chicago region of northeastern Illinois, and are < 15 km from one other. The Wadsworth Prairie, Lyons Woods, and Abbott Park populations are among the largest in Illinois, with plants occurring in successional prairie and sedge meadow (U.S. Fish and Wildlife Service 1999). All seeds were collected from mature capsules prior to dehiscence in late August or early October, and were stored at 5°C in a desiccator (containing

CaSO₄) prior to sowing. The mycorrhizal fungus used in germination treatments has been tentatively identified as a species of *Ceratorhiza* (L. W. Zettler, pers. obs.), and was isolated from the roots of a mature *P. leucophaea* specimen obtained from Abbott Park in 1995, and cultured at the Morton Arboretum.

Crossing effects on seed viability. This study investigated whether manually placing entire pollinia on stigmatic surfaces (= hand pollination) yielded a greater percentage of viable seeds than natural pollination. Hand pollination consisted of crosses between plants within populations, while natural pollination could also have included selfing through geitonogamy. Pollinated plants were not bagged to exclude subsequent natural pollination because placement of the entire pollinium on the stigma excludes additional pollen deposition. Pollinations were conducted in 1998, with one or two mature capsules sampled per plant from ten naturally pollinated plants and from nine hand-pollinated plants (Table 1). Seeds were pooled from capsules within plants, and approximately 200 seeds were sampled per plant. Seeds were briefly surface disinfested in dilute NaOCl, moist stratified for 11 mo. by suspending in sterile deionized water (SDW) at 6°C, and sown on a 1 × 4 cm filter paper strip in a 9 cm diameter petri dish containing 20 ml of modified oats medium (Dixon 1987). The dishes were then examined with a dissecting microscope to count numbers of apparently viable and non-viable seeds based on the presence or absence of distinct, rounded and hyaline embryos (Zettler et al. 2001). Viable seed numbers were expressed as a percent of the total seeds in each sample. These percentages were arcsine-transformed (Zar 1974), and tested against the null hypothesis that hand-pollinated capsules did not contain a greater percentage of viable seeds. We used a one-tailed t-test based on our expectation of more viable seeds with hand pollination because it maximizes pollen availability.

A second study examined crossing effects on production of viable seed using the Wadsworth Prairie and Abbott Park populations. We compared self-pollination (1 Wadsworth plant and 1 Abbott plant), outcrossing within populations (1 Abbott plant and 3 Wadsworth plants), and reciprocal outcrossing between populations (2 plants). These pollinations included > 5 flowers per inflorescence, and were conducted in 1997 (Table 1). Seeds collected from mature capsules were pooled within each plant, dis-

infested, scarified by shaking in 0.5% NaOCl for one hr., and stratified for 8 wk. at 5°C in SDW. Seed suspension samples were removed from stratification with an eyedropper. Each sample contained about 100 seeds, with ≥ 10 samples per cross. As described above, the numbers of seeds containing round distinct embryos were counted with a dissecting microscope and expressed as a percent of the total seeds. One of the selfed plants did not produce mature capsules with seeds, resulting in 0% viable seeds for this cross. Differences between crossing treatments were analyzed by inspection because the non-normal distribution of data and unbalanced design prevented appropriate statistical testing of the hierarchical nesting of seed sources within treatments.

Stratification and symbiotic culture effects on germination. In these experiments, we tested effects of duration of moist stratification, seed age (storage time), and presence or absence of fungal inoculant on seed germination. Seeds were disinfested and scarified in 0.5% NaOCl for 1 hr. We used seedling development stages as defined by Hadley (1983), where Stage 1 germination is achieved by rupturing of the testa (seed coat) by the enlarging embryo, and Stage 2 germination coincides with enlargement of the protocorm beyond the original seed size and development of rhizoids. Our observations suggest that scarification may promote Stage 1 germination by facilitating water imbibition and rupture of the testa by the enlarging embryo. In contrast, other studies (e.g., Zettler and Hofer 1998; Zettler et al. 2001) using unscarified seeds describe germination to Stage 1 as production of rhizoids, and Stage 2 as rupture of the testa. In this situation, unscarified seeds may initiate germination by producing rhizoids that help imbibe water and then cause rupture of the testa.

The effect of stratification period on germination to Stage 1 was tested on seeds collected in 1996 (Table 1). After scarification, seeds were plated on sterile filter paper moistened with SDW and given treatments of either no stratification (Abbott population), or moist stratification in darkness at 4°C for 8 wk. (all sites) or 16 wk. (Wadsworth and Lyons populations). Seed numbers ranged from 450 to 1140 per population. Seeds were plated onto modified oats medium in petri dishes, with six to nine replicates per treatment and 30–160 seeds per dish. Petri dishes were

wrapped in foil and incubated at 25°C in darkness, and germination was monitored biweekly for 11 mo. with a dissecting microscope. Additional germination that might have occurred with a second cold treatment was not considered in this experiment. As described above, seed viability counts were based on the presence of round distinct embryos. Counts of Stage 1 germinated seeds were pooled among replicate plates within each treatment and tested by Chi-square analysis for differences in numbers of germinated and ungerminated seeds among stratification periods, and between the Wadsworth and Lyons seed sources.

Effects of inoculant, stratification, and seed storage time on germination to Stage 2 were tested between the 1995 seed batch ($n = 3621$ seeds), which was stored for 18 mo., and the 1996 seed batch ($n = 1315$ seeds), which was stored for 6 mo. (Table 1). In this study, only scarified seeds were used, and seeds were pooled among seed sources. To test whether a fungal inoculant and stratification resulted in greater germination than either treatment separately, replicate plates for the 1995 and 1996 seed batches were given treatments of 16 wk. stratification, 16 wk. stratification plus inoculant, or inoculant with *Ceratorhiza* sp. As above, seeds were plated onto modified oats medium and seed viability counts were based on presence of distinct embryos. Germination was monitored biweekly for 11 mo. The number of Stage 2 seedlings on each plate was expressed as a percent of the number of viable seeds originally present on the plate.

A two-factorial ANOVA was used to compare germination treatment and seed storage time effects on percent germination. Exclusion of contaminated plates resulted in an unbalanced experimental design (replicates ranged from 4–16 plates), which we tested using a General Linear Model. Prior to analysis, all percentages were arcsine transformed (Zar 1974). We also tested for a correlation between the percentage of viable seeds and the percentage of those seeds reaching Stage 2. To determine whether seedling development (and seed viability) was independent of seed density, we tested for a correlation between the percentage of viable seeds reaching Stage 2 and the total number of seeds (both viable and non-viable) in each plate.

RESULTS

Crossing effects on percent viable seed. Seed viability varied among pollination crossing treatments made in 1997 and in

Crossing Effect on Percent Seed Viability

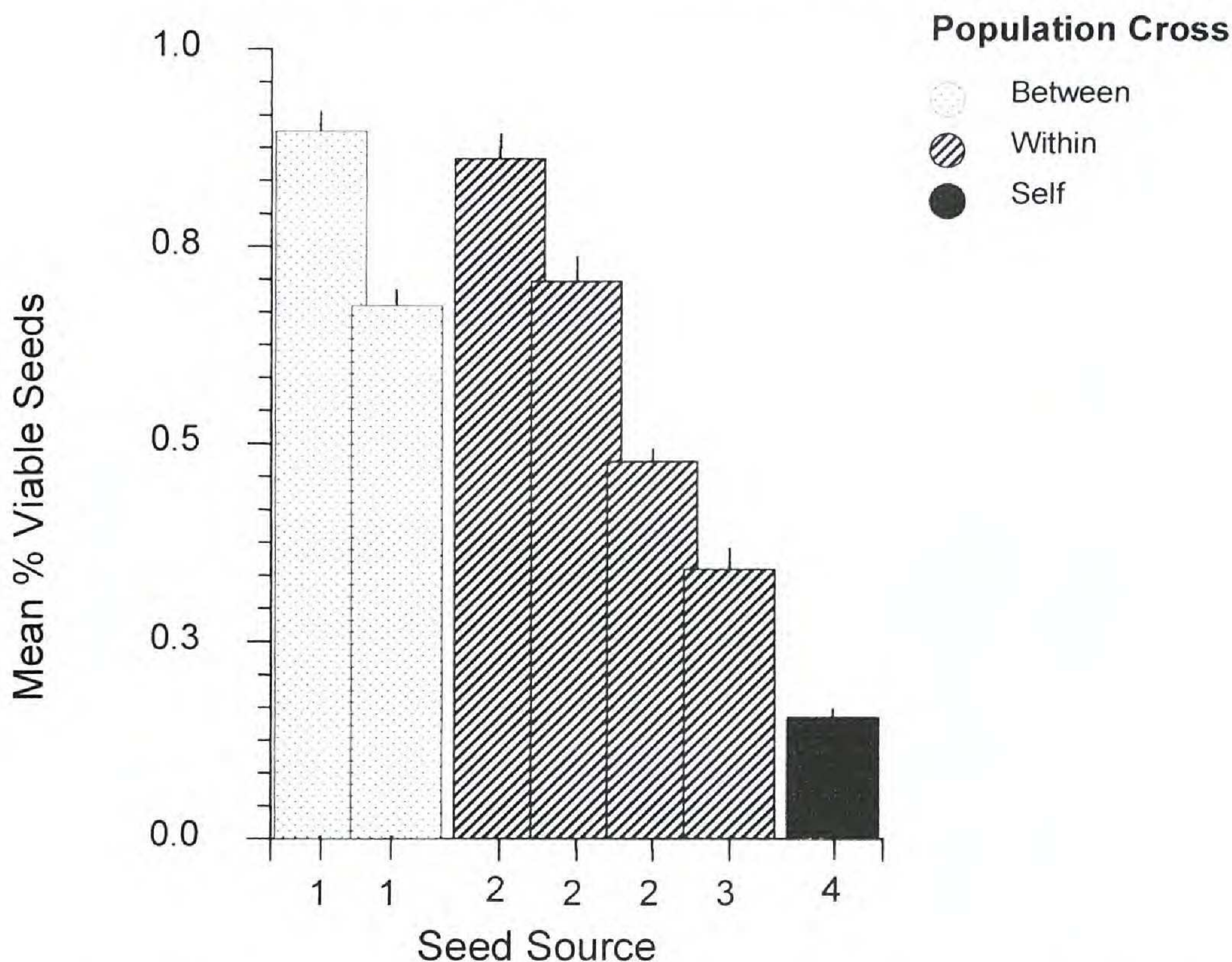


Figure 1. Differences in mean percent viable seed produced by selfing, crossing within, or crossing between populations of *Platanthera leucophaea*. Seed source replicates: 1 = Wadsworth \times Abbott, 2 = Wadsworth \times Wadsworth, 3 = Abbott \times Abbott, 4 = Abbott selfed. One Wadsworth selfed plant produced no viable seed. Lines represent standard errors.

1998. About 50% of the seeds in capsules obtained from hand cross-pollinations made in 1998 contained viable embryos, almost twice the percentage from naturally pollinated plants ($t = -1.785$, $P = 0.046$). Within-population outcrosses made in 1997 also averaged about 50% viable seed, but between-population crosses averaged almost 70% with wide variation among means (Figure 1). One self-pollinated plant produced no capsules with viable seeds, while the second averaged 15% viable seeds.

Effects of stratification period on Stage 1 germination. Stratification period, but not seed source, significantly affected germination to Stage 1, with percent germination increasing with increasing stratification period across all seed sources (Figure 2). Overall, germination was $< 5\%$ for unstratified seeds,

Stratification Period and Seed Source Effects on Seed Germination

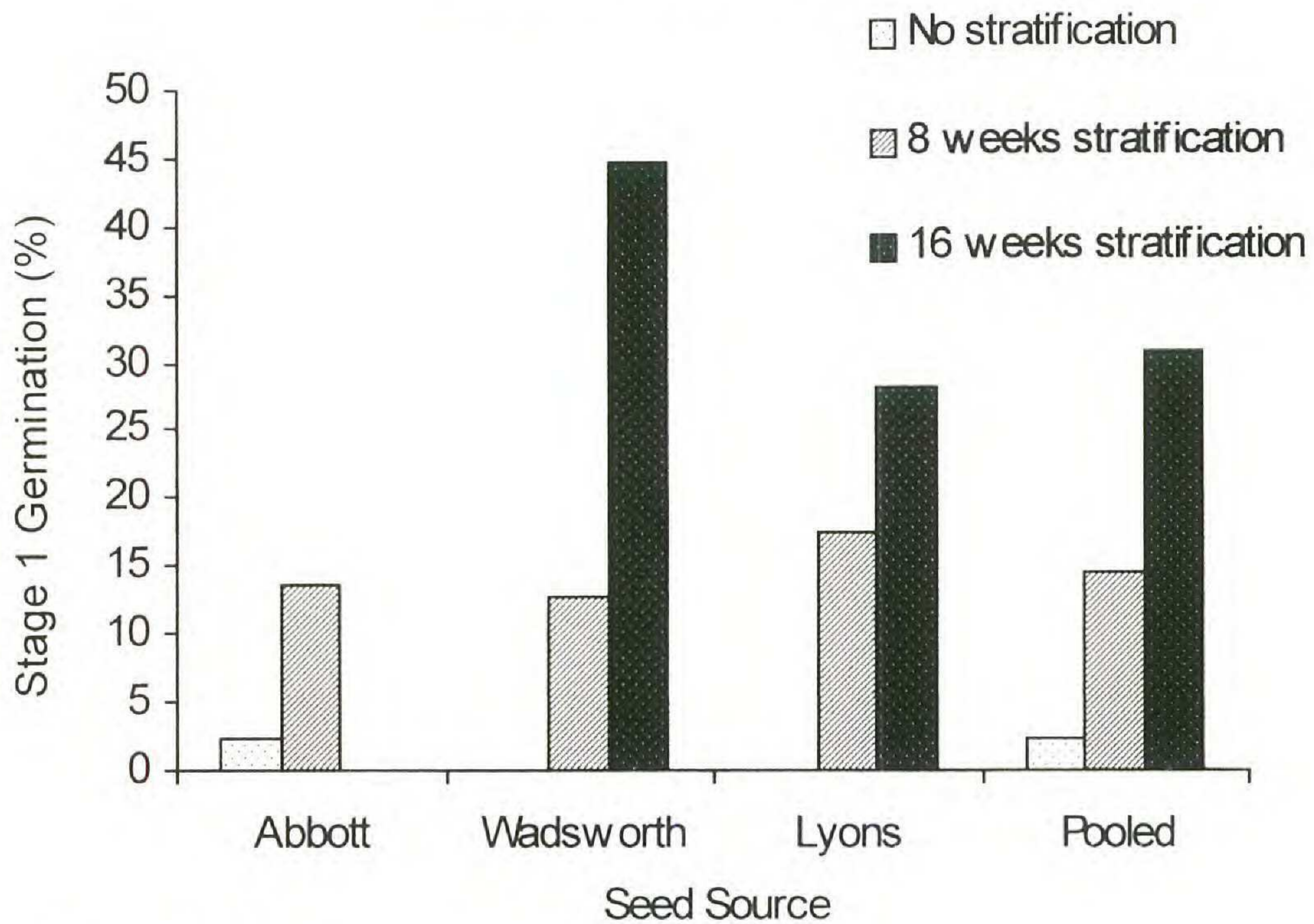


Figure 2. Longer stratification period increases percent seed germination of *Platanthera leucophaea*, with similar effects among seed sources. Chi-square: Stratification period ($\chi^2 = 275.76$, $P < 0.001$), Seed source ($\chi^2 = 0.945$, $P = 0.332$).

10–20% after 8 wk. stratification and $> 30\%$ after the 16 wk. stratification.

Effects of seed storage time, germination treatment, and seed viability on germination to Stage 2. No significant effects of seed storage time or germination treatment were found for Stage 1 germination. However, Stage 2 germination (rhizoid production) was significantly higher for seeds from the 1996 seed batch than for seeds collected in 1995 and stored for an additional 12 mo. (Figure 3). Moreover, Stage 2 germination in both seed batches was higher for stratified seeds that were also germinated symbiotically with *Ceratorhiza* than for either treatment alone (Figure 3). Among the 1996 seeds, percent germination to Stage 2 was also significantly correlated with percentage of viable seeds (Figure 4). Percent germination was not, however, significantly correlated with total seed number per plate ($r^2 = 0.003$, $P = 0.85$)

Treatment and Seed Age Effect on Germination

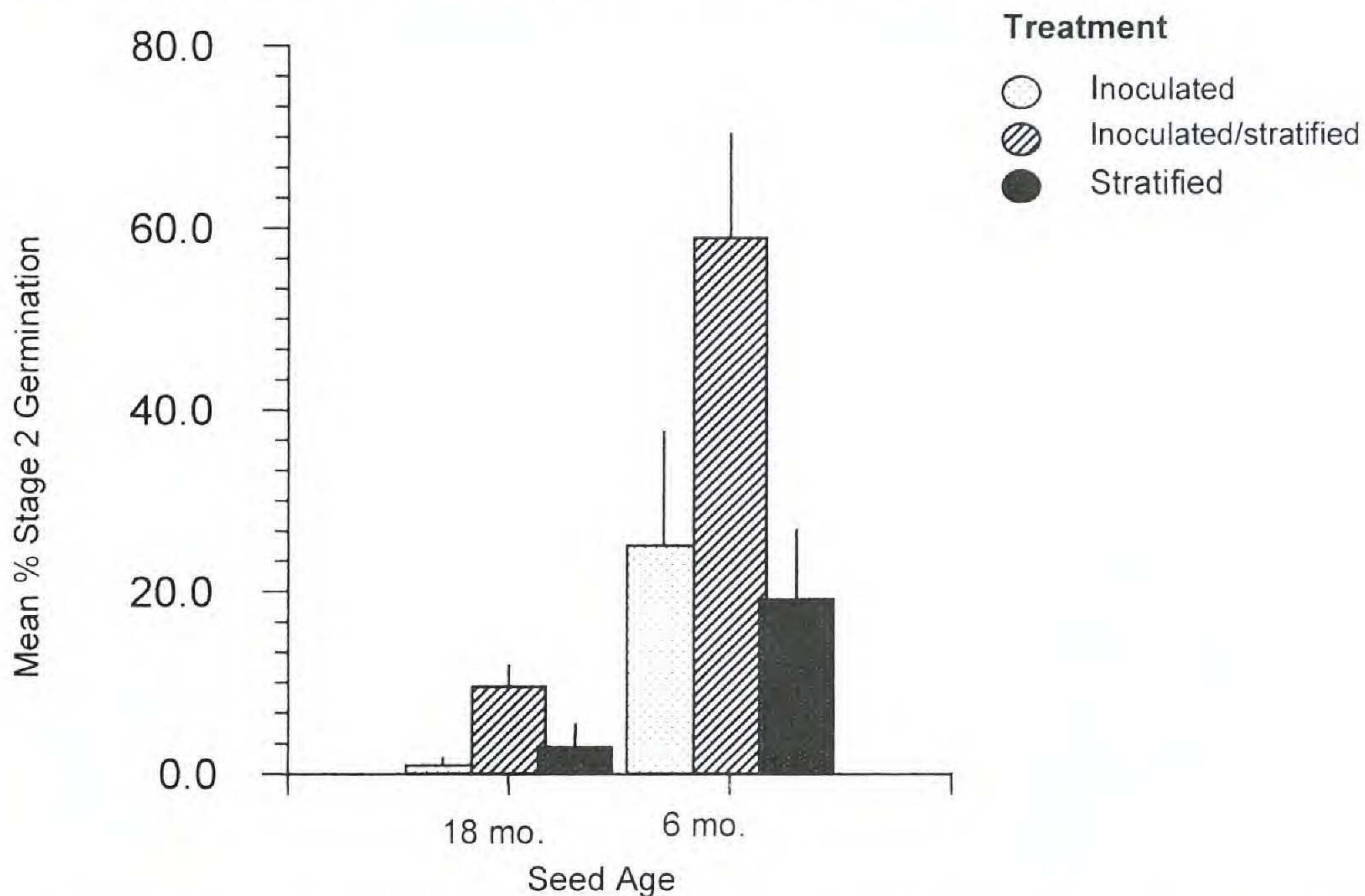


Figure 3. The mean percentage of viable *Platanthera leucophaea* seeds germinating to Stage 2 is lower for older seeds and greater for stratified seeds inoculated with *Ceratorhiza* sp. isolated from *P. leucophaea*. ANOVA: Treatment ($F = 6.97$, $P = 0.003$), Age ($F = 20.74$, $P < 0.0001$), Treatment \times Age ($F = 1.78$, $P = 0.1846$). Lines represent standard errors.

nor with total number of viable seeds per plate ($r^2 = 0.008$, $P = 0.755$).

DISCUSSION

Crossing effects on seed viability. As suggested for showy *Platanthera* (Gregg 1990) our pollination experiments indicate that *P. leucophaea* has a facultative outcrossing breeding system. Because this system allows mixed mating, it is apparently vulnerable to inbreeding depression, which can be expressed at different plant life-history stages (e.g., Carr and Dudash 1996; Dudash 1990; Fenster and Dudash 1994). In *P. leucophaea*, inbreeding depression appears to have cascading effects by decreasing the percentage of capsules formed, the percentage of viable seeds within capsules, and the percent germination of those seeds. For outcrossing species, this process may be alleviated in larger populations that maintain high levels of genetic diversity (Schaal et

Relationship Between Viable Seeds and Germination

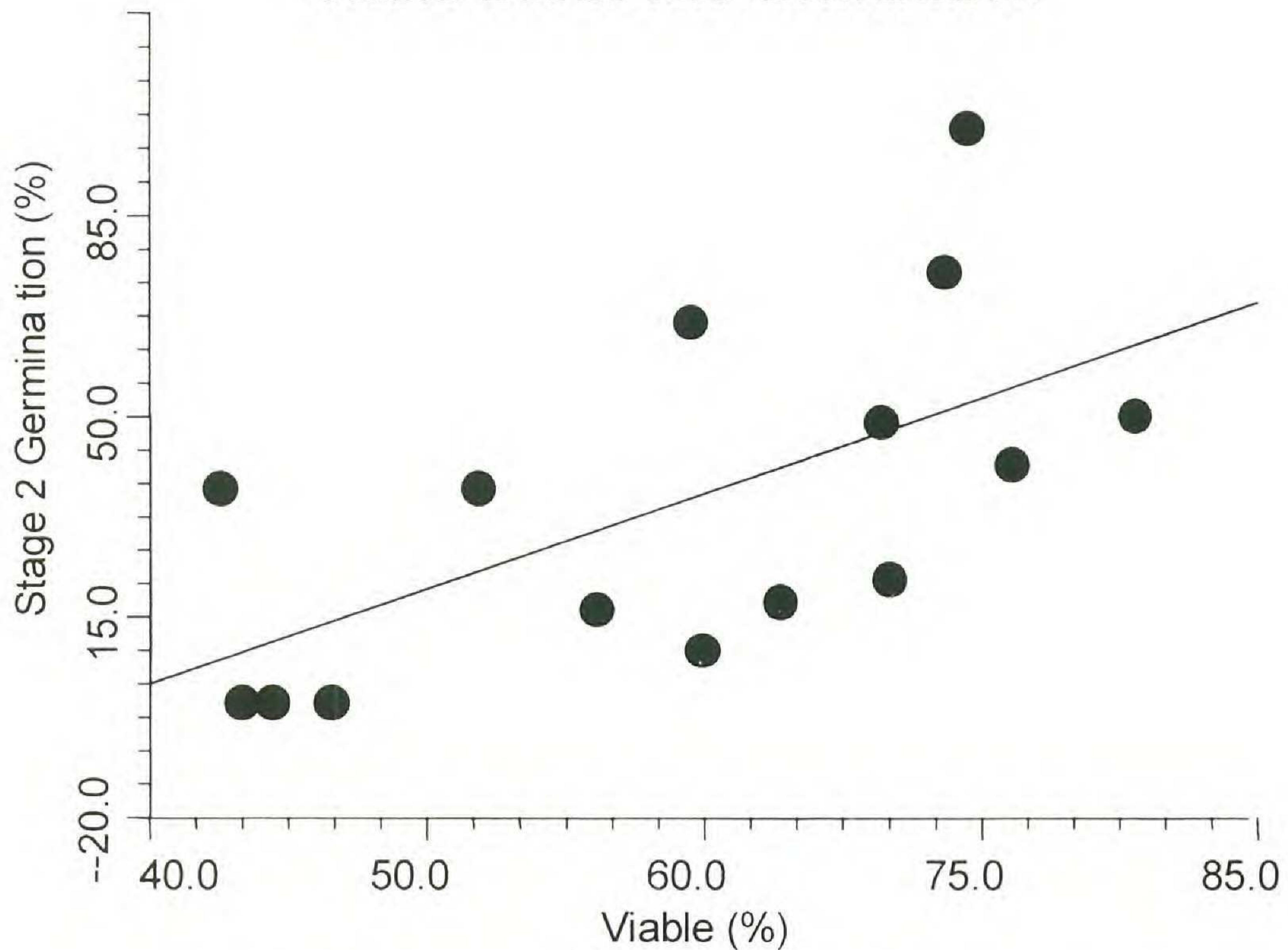


Figure 4. Within plates, the percentage of viable *Platanthera leucophaea* seeds germinating to Stage 2 is positively correlated with the total percentage of viable seeds ($r^2 = 0.374$, $P = 0.015$).

al. 1991; Weller 1994). The amount of inbreeding in small *P. leucophaea* populations could therefore be greater than in large populations because opportunities for outcrossing may be less in small populations. In our study, the lower percentage of viable seeds in naturally pollinated plants than in hand-pollinated plants may have resulted from inbreeding due to geitonogamy and crossing among closely related individuals, as well as from low rates of pollen deposition by hawkmoths. The wide variation we detected in seed viability among outcrosses within populations could reflect different levels of inbreeding based on chance.

Stratification and fungal symbiont effects on germination. *Platanthera leucophaea* seed germination is highly responsive to both stratification time and the presence of a fungal symbiont. As indicated by Stoutamire (1996), optimum germination requires a sequential combination of scarification and

moist stratification. Although all of our seeds were scarified, we found extremely low (< 5%) germination without stratification. Further, increasing stratification time from 8 to 16 wk. more than doubled germination from < 15% to > 30%. This suggests that for north temperate orchids, which may have evolved under selective pressure of cold dormant season conditions, both scarification and long-term stratification are necessary to attain high rates of seed germination.

Seed germination experiments that do not include sufficiently long moist stratification periods coupled with scarification and a fungal inoculant could lead to improper conclusions about seed germinability (Rasmussen 1995). For example, although north temperate species seem to depend on mycorrhizal fungi for seedling development (Johansen and Rasmussen 1992), variable results are reported. Zettler and McInnis (1992) found higher germination for *Platanthera integrilabia* with inoculated seeds, but Stoutamire (1996) reported that initial seedling germination for *P. leucophaea* did not require a fungal symbiont if cultured on artificial media containing a carbon source. Our results, along with those of others (e.g., Zettler and Hofer 1998; Zettler and McInnis 1992) underscore the importance of a fungal inoculant for successful germination in *Platanthera* species. The effect was especially apparent for seedling development to Stage 2 germination, which was maximized by the combination of 16 wk. stratification and presence of a fungal inoculant.

Seed age and storage techniques are also important factors in orchid seed germinability (Seaton and Hailes 1989). Stoutamire (1996) reported complete loss of *Platanthera leucophaea* seed viability after 6 mo. of storage at 5°C. Our results were less drastic, but similar, in that about 60% of the seeds reached Stage 2 germination after 6 mo. of storage at 5°C, but only 10% reached Stage 2 after 18 mo. of storage. However, Zettler (1996b) reported viable *P. integrilabia* seeds after 6 yr. of storage at -7°C and 6°C. Loss of viability may be related to failure to adequately dry seeds prior to and during storage (L. W. Zettler, pers. obs.), complex dormancy mechanisms (Johansen and Rasmussen 1992), and different species characteristics.

For north temperate *Platanthera*, including *P. leucophaea*, propagation beyond Stage 2 may be accompanied by high rates of mortality, especially if using an aggressive fungal symbiont (Zettler and Hofer 1998; Zettler and McInnis 1992; Zettler et al.

2001). As a result, symbiotic orchid propagation without host-specific fungi may be problematic. Screening of *P. leucophaea* fungal inoculants from naturally germinating seeds and seedlings could help alleviate this problem. Research is also needed to determine whether the combination of proper scarification, stratification, inoculation, and secondary cold treatments can enhance the transition from Stage 2 germination to further leaf and tuber development (Johansen and Rasmussen 1992), and how pollination outcrossing rates affect this transition.

Conservation applications and concerns. Although *Platanthera leucophaea* is perennial, most individuals flower once, and seedling establishment appears to be a critically important stage in its life cycle (Bowles and Bell 1999). Thus, factors that increase production of viable seeds should enhance population viability. In that regard, hand pollination may be an important tool because it can increase viable seed numbers by maximizing pollen deposition and avoiding inbreeding. Hand crossing among fragmented populations appears most likely to enhance viability, but it is controversial because of concerns that outbreeding depression may result from the disruption of locally adapted gene complexes (Bowles and Whelan 1994). For example, genetic allozyme (Cowden 1993) and random polymorphic DNA (Havens and Buerkle 1999) studies of *P. leucophaea* have found comparatively high levels of genetic differentiation among populations, which indicates potential for outbreeding depression. However, human-caused population fragmentation and reduced gene flow could have contributed to such differences, and it is unknown whether outbreeding depression would actually occur. For example, Fenster and Galloway (2000) found outbreeding depression to be important in *Chamaecrista fasciculata* Michx. only for crosses of ≥ 1000 km, a distance far greater than among our study sites. Hawkmoths are well known for long-distance movement, which may have facilitated landscape-scale gene flow in *P. leucophaea* that would have tended to minimize population differentiation. Human-mediated crosses can alleviate potential inbreeding within fragmented populations of outcrossing species (Richards 2000). For example, heterosis from long-distance crosses has been observed in the orchid *Liparis lilifolia* (L.) A. Rich. ex Lindl. (Whigham and O'Neill 1991), and such crosses have been used to obtain viable seed of the orchid *Cypripedium cal-*

ceolus L. var. *pubescens* (Willd.) Correll in Britain (Light and MacConaill 1998). Our results indicate this may be possible for *P. leucophaea*, as the greatest percentage of viable seeds resulted from inter-population crosses.

Another concern is that high levels of seed production from hand pollination could impose a significant cost on terrestrial orchids, as found for *Tipularia discolor* (Pursh) Nutt. (Snow and Whigham 1989) and *Cypripedium acaule* Aiton (Primack and Hall 1990). Calvo (1993) argued that the naturally low rates of orchid seedling recruitment would not select for increased pollination and seed production. Kull (1998) also found that microsite factors, rather than pollinators, limited population growth in the long-lived perennial *C. calceolus*. However, such effects may be less important in short-lived orchid species. The short life span of *Platanthera leucophaea*, its lack of vegetative spread, and its showy, and apparently costly, inflorescence structure suggest that high rates of seed production, and more importantly, high levels of seed viability, are important for population maintenance in this species. Ultimately, successful seedling establishment will depend upon chance coupling of germinating seeds with hyphae of favorable soil fungi, and rates of this demographic process remain essentially unknown for terrestrial orchids.

These concerns, and our crossing experiments, indicate that further work is needed to assess the impacts of translocating genetic material among populations, and whether there are negative demographic consequences of increased rates of pollination. Fully replicated crossing experiments are also needed to test for plant and site effects on seed viability.

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